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Studies on a Factor Enhancing Colicin E3 Activity *In Vitro*

(ribosomes)

YOSHINORI OHSUMI AND KAZUTOMO IMAHORI

Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo, Japan

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ABSTRACT **The mechanism of action of colicin E3 (E3) was investigated in an *in vitro* system. Purified ribosomes are less susceptible to E3 than crude or washed ribosomes. A factor was found in the supernatant fraction of normal *Escherichia coli* cells that stimulates inactivation of ribosomes by E3, and on addition of this factor, about one tenth as much E3 was required for inactivation of ribosomes. On heating a mixture of E3 and this factor above 60°, the ribosome inactivating activity of E3 increased greatly, and an amount corresponding to 0.01 μg of E3 was sufficient to inactivate 1.0 A_{260} unit of ribosomes completely. By this treatment bacteriocidal activity of E3 decreased considerably, as the ratio of the two activities of E3 (ribosome inactivating activity and bacteriocidal activity) increased to 6×10^4-fold. It is evident that the two activities do not run in parallel.**

This heat-treated product cleaved 16S rRNA in the same way as E3. These results suggest that inactivation of ribosomes is not due to colicin molecules prepared by the standard procedure, but to a modified form of them.

Colicin E3 (E3) is a protein antibiotic produced by certain strains of coliform bacteria that carry a specific episome (1). The adsorption of E3 to sensitive bacteria causes marked inhibition of protein synthesis, due to inactivation of 30S ribosomal subunits (2, 3). It has also been shown that the inactivation of 30S subunits is due to cleavage of 16S rRNA at a specific position near the 3′-terminus (4, 5). Recently, it was found that both ribosome inactivation and cleavage of 16S rRNA can be achieved *in vitro* (6, 7). This indicates that E3 molecules penetrate the cells and interact directly with target ribosomes, thus, cleaving RNAs without the aid of other cellular components. But in *in vitro* systems, the amount of E3 required for ribosome inactivation or the cleavage reaction, is much larger than the amount of enzyme required for most enzymatic reactions.

Previously, one of us reported that the supernatant fraction of E3 infected cells can inactivate ribosomes in an *in vitro* system (8). In this system, cells infected with E3 were treated with trypsin to digest excess colicin. Then trypsin inhibitor was added and the cells were disrupted by sonication. Then the S100 fraction was separated (called i-S100). It was found that i-S100 retained ribosome inactivating activity, but did not show the lethal activity, characteristic of colicin. In the same report it was indicated that normal *Escherichia coli* cells contain some substance that stimulates the inactivation reaction of E3 *in vitro*. These results suggested that intact colicin itself might not inactivate ribosomes.

This paper describes further studies on the action of this factor and shows that E3 prepared by the standard method might not participate in cleavage of rRNA.

Abbreviation: E3, colicin E3.

MATERIALS AND METHODS

Colicin E3. E3 was induced in strain W3110 (E3), obtained from Dr. Helinski, by treatment with Mitomycin C (0.5 μg/ml), and was purified by successive chromatographies on DEAE-Sephadex A-50 and CM-Sephadex columns, as described by Herschman and Helinski (9). The final preparation was stored as a solution of 1 mg or 3 mg/ml at −80°. This material gave a single band on polyacrylamide gel electrophoresis.

Preparation of Ribosomes. Cells of strain K12-A19 (met$^-$, RNase I$^-$) were harvested in the log phase of growth and 50 g (wet weight) of cells were ground with alumina and extracted with 100 ml of buffer A (10 mM Tris·acetic acid at pH 7.8, 10 mM magnesium acetate, 50 mM NH_4Cl, and 6.5 mM mercaptoethanol) with a little of DNase. Ribosomes were prepared from the S30 fraction by centrifugation for 4 hr at 105,000 × *g* in a Hitachi, RP65 rotor. The top half of the supernatant was used as the S100 fraction for protein synthesis. Then the ribosomes were washed twice in buffer B (20 mM Tris·acetic acid at pH 7.8, 10 mM magnesium acetate, 1 M NH_4Cl). The final pellet, after suspension in buffer C (buffer A without mercaptoethanol) and dialysis against the same buffer (washed ribosomes) were applied to a column of DEAE-Sephadex A-50 equilibrated with buffer C and washed thoroughly with buffer C supplemented with 0.25 M NH_4Cl. Then the column was eluted with buffer C supplemented with 0.45 M NH_4Cl. Ribosome fractions were pooled, precipitated by centrifugation at 133,000 × *g* for 4 hr, suspended in buffer C and stored at −80° (purified ribosomes).

Preparation of the Factor. A sample of 40 g of K12-A19 cells were suspended in 100 ml of buffer C and disrupted by sonication for 20 min. The preparation was clarified by low-speed centrifugation and then centrifuged at 105,000 × *g* for 4 hr. The supernatant was incubated in boiling water for 5 min. The precipitate was removed by centrifugation, and the resulting supernatant was dialyzed against buffer C overnight at 4°. Then it was charged on a DE-52 column (Whatman, 4 × 10 cm) equilibrated with buffer C and eluted with the same buffer. Fractions eluted in the peak with an absorbancy at 280 nm were pooled, mixed with 2 volumes of cold ethanol and kept in a deep freeze for 1 hr. The precipitate was collected by centrifugation, suspended in buffer C, and dialyzed against the same buffer. The final solution (factor) had an absorption maximum at about 279 nm. Its absorbance at this wave length was about two.

In Vitro E3 Assay. Ribosome inactivating activity was assayed by the following two steps: preincubation of ribo-

somes with E3, E3 plus the factor, or a heated mixture of the two for the desired period (inactivation step), and then measurement of residual ribosome activity, as ploy(U) directed phenylalanine incorporation (incorporation step).

(*a*) Inactivation step: Samples of 1.0 A_{260} unit of ribosomes were incubated with various amounts of E3 plus the factor at 37° for 5–60 min in a total volume of 30 μl of buffer C supplemented with 10 mM $NaBH_4$. Then the mixture was chilled.

(*b*) Incorporation step: To the above mixture of ribosomes and E3 were added 50 μl of assay mixture. The resulting reaction mixture in a volume of 80 μl contained: 20 mM Tris·acetic acid at pH 7.8, 15 mM magnesium acetate, 100 mM NH_4Cl, 1 mM GTP, 4 mM ATP, 6 μg poly(U), 5 mM creatine phosphate, 2 μg of creatine kinase, 3 μl of S100, 0.1 μCi of [^{3}H]L-phenylalanine (1 mCi/mM). The reaction mixtures were incubated at 37° for 1.5 min. Reactions were stopped by adding 3 ml of 5% trichloroacetic acid. Hot trichloroacetic acid-insoluble materials were collected on glass fiber filter (Whatman GF/83) and counted in toluene-base scintillation fluid.

Under these assay conditions, protein synthesis was limited by ribosomes. Moreover, since incorporation proceeded linearly for at least 2.5 min, synthesis was directly proportional to the number of active ribosomes. A reaction time of 1.5 min was chosen to minimize the complication of inactivation during the incorporation step.

Preparation of ^{32}P-Labeled Ribosomes. E. coli K12-A19 cells were grown at 37° in 0.6 l of medium containing 12.1 g of Tris, 0.5 g of casamino acids (Difco), 1.0 g of glucose, 23 mg of Na_2SO_4, 120 mg of $MgSO_4$, 155 mg of $CaCl_2$, 0.5 mg of $FeCl_3$, 7.9 mg KH_2PO_4, and 50 mg of L-methionine per liter. Then they were labeled by the addition of carrier-free $H_3{}^{32}PO_4$ (10 mCi) for 3 hr and harvested. Unlabeled cells, grown separately in 3.0 ml of the same medium supplemented with 44 mg of KH_2PO_4 per liter, were combined with the labeled cells and washed twice with buffer C. ^{32}P-Labeled ribosomes were obtained from the cells as described previously. The final suspension showed 200 A_{260}/ml and 1.0 × 10^6 cpm/A_{260} unit.

Polyacrylamide Gel Electrophoresis. Samples of 1.0 A_{260} of ^{32}P-labeled ribosomes were incubated at 37° for 30 min with E3, E3 plus factor, or a heated mixture of the two in 30 μl of buffer C. Then, final concentration of sodium dodecyl sulfate, EDTA, and sucrose were added to a final concentration of 0.1%, 2 mM, 16%, respectively, with bromphenol blue. After incubation for 15 min at 37°, 15-μl samples from 50 μl of each mixture were applied to 12% polyacrylamide gel (100 × 145 × 1.5 mm) and subjected to electrophoresis in Tris·glycin buffer at pH 8.6 (5.9 g of Tris and 29 g of glycin per liter) for 4 hr at 100 V and 4° to separate rRNAs, following the method of Meyhack *et al.* (10). Then the gel was exposed to x-ray film for 24 hr.

Materials. ATP, GTP, creatine phosphate, poly(U), creatine kinase, trypsin, and soybean trypsin inhibitor were purchased from Böehringer, Mannheim. [^{3}H]L-Phenylalanine (1.0 mCi/mM) was purchased from New England Nuclear Corp.

RESULTS

Susceptibility of purified ribosomes to E3

There are many reports that even after very extensive washing with solutions of high salt or sucrose concentration, ribosomes remain susceptible to E3 (10). On the other hand, it is known that ribosomes can bind various nonribosomal materials tightly, and that DEAE-Sephadex column chromatography is fairly effective for removing such contaminants.

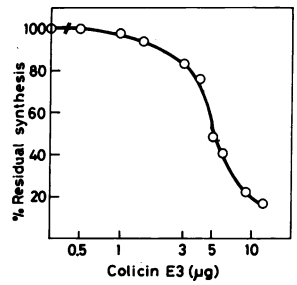

FIG. 1. Effect of E3 on purified ribosomes. Samples of 1.0 A_{260} of purified ribosomes were incubated at 37° for 30 min with the amounts of E3 shown on the abscissa in 30 μl of buffer C supplemented with 10 mM $NaBH_4$. Then the residual ribosome activity was assayed as described in *Methods* (Incorporation step). Values are percentage of those of sample which were not incubated with E3.

Ribosomes, purified by DEAE-Sephadex column chromatography, were less susceptible to E3 than crude or washed ribosomes. However, they are not completely insensitive to E3, and also lose activity when incubated with a large amount of E3 (Fig. 1). Purified ribosomes have almost the same activity as washed ribosomes for poly(U)-directed phenylalanine incorporation, and have normal fMet-tRNA binding activity when initiation factors and R17-RNA or AUG are supplied. Therefore, these ribosomes do not seem to be deficient in ribosomal components, or to have sustained conformational damage. Purified ribosomes were mainly used in the present experiments, but similar results were obtained when washed ribosomes were used. The low susceptibility of purified ribosomes to E3, suggested the existence of some factor which stimulated the action of colicin. In support of this it was found that the unadsorbed fraction obtained from the DEAE-Sephadex column during purification of ribosomes stimulated colicin activity *in vitro*. The supernatant fraction of normal cells disrupted by sonication had a similar effect. We tentatively named the material which stimulated colicin activity in this supernatant fraction a factor.

Effect of the factor on the inactivation step of *in vitro* colicin assay

The factor stimulated the ribosome inactivating activity of E3 *in vitro*, though it had no effect on ribosomes when added alone (Fig. 2). The incorporating activity of 1.0 A_{260} of purified ribosomes was not affected by incubation with 1.5 μg of E3 alone, but on addition of the factor in the inactivation step, activity first decreased in proportion to the amount of the factor added and then began to level off at high concentration of the factor. On altering the amount of E3 with a saturating amount of the factor, it was found that the amount of E3 required for 70% inactivation of 1.0 A_{260} unit of ribosome during 60-min incubation could be reduced to 0.3 μg.

The factor was very heat stable, and its activity was stable on heating for 5 min at temperatures of up to 100°. The unadsorbed fraction obtained by DEAE-Sephadex column chromatography during purification of ribosomes was also heat-stable.

For inactivation of ribosomes by E3 and the factor, a reducing agent, such as dithiothreitol, sodium borohydride, or

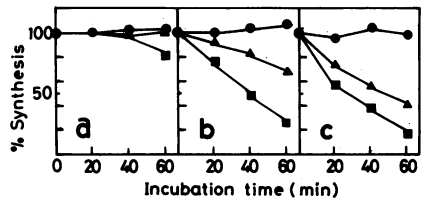

FIG. 2. Stimulation of E3 activity by the factor. Samples of 1.0 A_{260} of ribosomes were incubated at 37° with or without E3 and with or without the factor for the times shown on the abscissa in a total incubation mixture of 30 μl (inactivation step in the *Methods*). The residual ribosome activity was assayed. (a) without the factor; (b) with 3 μl of the factor; (c) with 6 μl of the factor; ●, without E3; ▲, 0.3 μg of E3; ■, 0.75 μg of E3.

glutathione was required. The optimum concentration of reducing agent was rather high, e.g., 5 mM for dithiothreitol, or 10 mM for $NaBH_4$.

Effect of heating a mixture of E3 and the factor

Mixtures of E3 and two concentrations of the factor were divided in half. One half was kept at 0° as a control, and the other was heated at 67° for 5 min. The activity of each was then assayed in the *in vitro* system. Fig. 3 shows the results. With the control mixtures the incorporating activity of ribosomes decreased with increase in the amount of factor added. With heat-treated mixtures, the incorporating activity of ribosomes decreased within a few minutes to only a small percentage of the original activity. This level corresponded to the activity of ribosomes from E3-infected cells, and was well below the level attained with E3 alone or an unheated mixture of E3 and the factor. The inactivating effect of the heated mixture was also proportional to the amount of factor added. With excess factor, inactivation was so fast that the incorporation activity of ribosomes at zero time decreased, indicating that inactivation occurred during the short period of the incorporation assay. The temperature dependence of the inactivation of ribosomes was similar to that characteristic of a usual enzymic reaction, and they were even inactivated slowly at 0°. Results on temperature dependence of this heat-treatment (Fig. 4) show that the effect appeared at above 40°, and increased rapidly on increase in temperature to 60°. The optimum temperature was around 70°, and above 85° the

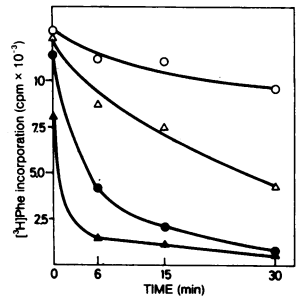

FIG. 3. The stimulation of E3 activity by heat-treatment with the factor. E3 (30 μg) was combined with 10 μl ○ or 40 μl △ of the factor in a total volume of 100 μl of buffer C. Each sample was divided in half. One half kept at 0° (empty symbols) and the other was incubated at 65° for 20 min (filled symbols). Then 5 μl of each sample was taken for assay of ribosome inactivating activity, as described in the *Methods*. (Abscissa represents the time in inactivation step).

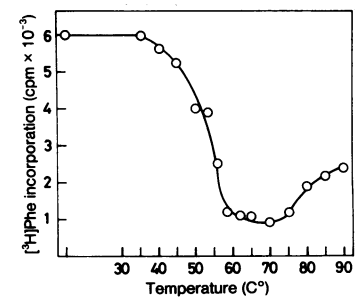

FIG. 4. Effect of temperature of heat-treatment on activity of a mixture of E3 and the factor. Mixtures of 3 μg of E3 and 80 μl of the factor in total volume of 90 μl of buffer C were incubated for 15 min at the temperatures indicated. Then the ability of 10 μl of each sample to inactivate ribosomes was measured as described in the *Methods* by carrying out the inactivation step for 15 min at 37°.

effect decreased. The decrease in the ribosome inactivating activity of the heat-treated mixture at very high temperatures may be due to heat denaturation of the active substance. E3 and the factor were heated separately and mixed after cooling. As shown in Fig. 5, the ribosome inactivating activity of the mixture was more than that of the unheated mixture but much less than that of E3 and the factor heated together (Fig. 5). As shown in Fig. 6, this enhancement of ribosome inactivating activity did not occur instantaneously even at 67°, which is above the denaturation temperature of E3, but proceeded gradually, reaching completion in about 60 min. These results suggest that E3 reacts with the factor at high temperature and that the effect of heat-treatment is not simply due to denaturation of E3 or the factor.

To examine the minimal requirement of E3 after heat-treatment with the factor, various amounts of E3 were heated

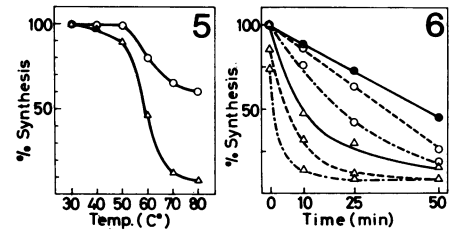

FIG. 5. (*left*) Effect of high temperature on E3 and, or the factor. Samples of 15 μg of E3, 25 μl of the factor, and a mixture of the two, each in total volume of 50 μl of buffer C, were heated for 5 min at the temperatures indicated. After chilling, the corresponding samples of E3 and of the factor were combined, while the mixture of the two was diluted 2-fold with buffer C. Then 10 μl of each sample was assayed as described in Fig. 4. ○, values when E3 and the factor were heated separately and then combined; △, values when E3 and the factor were mixed before heating.

FIG. 6. (*right*) Time course of heat-treatment. Samples of 30 μg of E3, 90 μl of the factor, and a mixture of the two, each in total volume of 110 μl of buffer C, were incubated at 67° for 5, 15, and 30 min and then chilled. Then the mixture of the factor and E3 was diluted 2-fold with buffer C, while the separate samples of E3 and the factor were combined. Ten-microliters of each sample were used to measure ribosome inactivating activity. (Abscissa represents the time in inactivation step.) ●, unheated control; ○, E3 and the factor heated separately and then combined; △, E3 and the factor heated together. Time of heating: ——, 5 min; – – –, 15 min; — — —, 30 min.

TABLE 1. *Activities of E3 after treatments*

	(a) Amount of E3 required (μg)	(b) Specific activity (1/μg)*	(c) Lethal activity (1/μg)	(d) b/c*
Purified E3	5	1	8.1×10^7	1
Purified E3 + factor (0°)	0.4	12.5	6.5×10^7	15.6
Purified E3 + factor (70°)	0.008	625	8.0×10^5	6.3×10^4

a: The amount of E3 required for reducing the activity of 1.0 A_{260} of purified ribosomes by half during incubation at 37° for 30 min.

c: See ref. 11 as for the definition of killing unit.

* The number in columns *b* and *d* are values relative to purified E3 used as a standard.

with a sufficient amount of the factor at 70° for 30 min and then their inactivating effects were measured. The results indicated that heat-treated product, corresponding to 0.004 μg of E3, reduced the activity of 1.0 A_{260} ribosomes by half in 40 min. Assuming that colicin molecules were activated by heat-treatment with the factor, the specific activity of E3 was increased several hundred fold by this treatment (Table 1). Heat-treatment with the factor greatly decreased the bacteriocidal activity of E3, and on treatment at 70° for 30 min, the lethal activity decreased to about 1% of that of the control incubated at 0°. The reducing agents mentioned above were not required during heat-treatment but were required in the ribosome inactivating step.

Action of the heat-treated product

To see if the action of the heat-treated product on 16S rRNA was the same as that of E3, we incubated the product with ^{32}P-labeled ribosomes at 37° for 30 min. Then sodium dodecyl sulfate and EDTA were added and the RNAs were analyzed on 12% polyacrylamide gel. The results are shown in Fig. 7. When a large amount of E3 was applied, a band corresponding to the E3-fragment was seen and its intensity was proportional to the amount of E3 applied. The heat-treated product also gave the same band, and the amount of E3 necessary for its formation was greatly reduced, 0.033 μg of E3 being enough to cause complete formation of the E3-fragment in 30 min. Therefore, the effect of the heat-treated product on the incorporating activity of ribosomes is due to specific cleavage of 16S rRNA.

DISCUSSION

It has been suggested that E3 requires a specific structure or conformation of ribosomes in its function (12–14). It is also known that reversible changes in ribosomal activity can be induced by relatively mild treatments and that these changes are accompanied by conformational changes (15). Thus, the factor may react with ribosomes and convert them into the forms sensitive to E3. However, the results of heat-treatment of a mixture of E3 and the factor indicate the direct interaction between the factor and E3. The structure of ribosomes may not be a major problem in the present experiments.

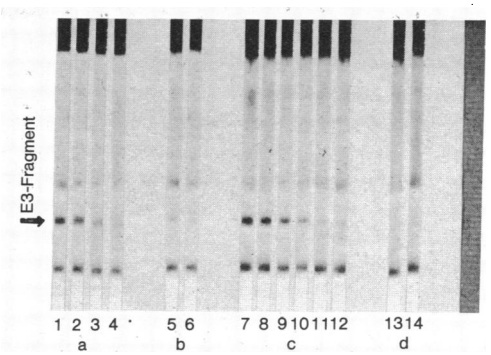

FIG. 7. Separation of the E3-fragment by polyacrylamide gel electrophoresis. Samples of 1.0 A_{260} of ^{32}P-labeled ribosomes were incubated at 37° for 30 min, with (a) E3 alone, (b) a mixture of E3 and the factor, or (c) a heat-treated mixture of E3 and the factor. Heat-treatment was carried out at 70° for 30 min. The amounts of E3 contained (a) 1, 10 μg; 2, 3.3 μg; 3, 1.1 μg; 4, 0.3 μg; (b) 5, 0.1 μg; 6, 0.033 μg; (c) 7, 0.1 μg; 8, 0.033 μg; 9, 0.011 μg; 10, 0.0037 μg; 11, 0.0012 μg; 12, 0.00041 μg; (d) 13, factor only; 14, buffer C only.

When E3 was heated with a factor its ribosome inactivating activity greatly increased, while its bacteriocidal activity decreased. As the results indicate, the ratio of the former to the latter increased 6×10^4-fold during this heat-treatment (Table 1). Thus it is evident that bacteriocidal and ribosome inactivating activities do not run in parallel. This is also supported by the following experiment. When E3 was heated alone, its bacteriocidal activity was rapidly lost above 65°, while its ribosome inactivating activity increased by the treatment at near 70°.

It is suggested that colicin E3 still plays some role even after heat-treatment with the factor, since we found that anti E3 serum inhibited the ribosome inactivating activity of the heat-treated product in proportion to the amounts added (results not shown). It should be emphasized that the product of the heat-treatment inactivated the ribosome completely in the same way as E3 but with remarkably high efficiency (Fig. 7).

For complete inactivation of purified ribosomes, 10 μg of E3 were required, corresponding to a molar ratio of E3 to ribosomes of 7.6. In the case of the heat-treated product, the ratio decreased at the most to 0.006. Based on the former value it is very difficult to explain the highly effective reaction *in vivo* and its single hit survival curve.

To explain the above results, at least two models can be proposed. (*1*) Colicin E3 prepared by the standard purification procedures still binds inhibitor, probably as immunity substance, tightly. On heat-treatment with the factor, inhibitor is removed and E3 gains ability to inactivate ribosomes. (*2*) Purified colicin E3 is free from immunity substance. E3 molecules have no ribosome inactivating activity by themselves, but after some modification they acquire this activity. This modification might involve conformational and chemical modification as well as fragmentation.

If model *1* is the case, almost all E3 must bind the immunity substance to explain the above results because the immunity substance seems to act on E3 not enzymatically but stoichiometrically (16, 17).

When E3 was treated with a low concentration of trypsin and then the trypsin neutralized with soybean trypsin inhibitor, the bacteriocidal activity was found to have de-

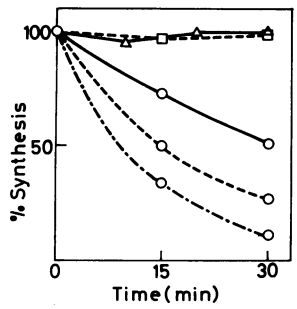

FIG. 8. Trypsin-treatment of E3. Mixtures of 60 μg of E3 and 1 μg of trypsin were incubated at 37° in 50 μl of buffer C. After 10 min, 1.25 μg of soybean trypsin inhibitor was added and incubation was continued for 5 min. Controls without E3 and with trypsin inhibitor added before incubation were also set up. Then, the samples were diluted and their ribosome inactivating activity and bacteriocidal activity were measured. By this treatment, bacteriocidal activity was reduced to about 10% of the original. △, trypsin and trypsin inhibitor (without E3); □, E3 (1.07 μg) treated with a mixture of trypsin and trypsin inhibitor at 37° for 10 min; ○, E3 treated with trypsin; the amounts of E3 were (——) 0.062 μg, (– – –) 0.19 μg, (— – —) 0.56 μg.

creased while the ribosome inactivating activity increased considerably (Fig. 8). This fact can be explained by the model *1* or *2*, but by any means, this suggests that the whole structure of E3 prepared by standard procedures is not required for its ribosome inactivating activity but rather the destruction of some part of the molecule is required to obtain the activity.

It is tempting to speculate that E3 molecules are bound to receptor on the cell surface and after some reaction are released into the cytoplasm in an active form as with diphtheria toxin (18). More detailed characterization of the product of heat-treatment and identification of the essential component in fraction i-S100 are required to elucidate the relation between bacteriocidal and ribosome inactivating action of E3.

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